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Capacity for Denitrification and Reduction of Nitrate to Ammonia in a Coastal Marine Sediment

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The capacity for dissimilatory reduction of NO_3^- to N_2 (N_2O) and NH_4^+ was measured in $^{15}NO_3^-$-amended marine sediment. Incubation with acetylene (7×10^{-3} atmospheres [normal]) caused accumulation of N_2O in the sediment. The rate of N_2O production equaled the rate of N_2 production in samples without acetylene. Complete inhibition of the reduction of N_2O to N_2 suggests that the "acetylene blockage technique" is applicable to assays for denitrification in marine sediments. The capacity for reduction of NO_3^- by denitrification decreased rapidly with depth in the sediment, whereas the capacity for reduction of NO_3^- to NH_4^+ was significant also in deeper layers. The data suggested that the latter process may be equally as significant as denitrification in the turnover of NO_3^- in marine sediments.

The reduction of NO_3^- to N_2 and to NH_4^+ is mediated by bacteria which are able to perform one or both of these reductive pathways.

In anoxic environments, the respiratory reduction of NO_3^- to N_2 is an alternative to O_2 respiration for the denitrifying bacteria. In rich organic sediments, e.g., those of many marine coastal areas, these conditions restrict the occurrence of denitrification to a zone immediately below the oxidized surface and reduced microniches within the surface zone.

The other pathway, the reduction of NO_3^- to NH_4^+, is a nutritional supplement for many bacteria if reduced nitrogen for assimilation is in short supply, but coastal marine sediments are most often rich in NH_4^+, and the assimilatory demands are readily met.

This reduction may be entirely dissimilatory, however, where the reduced product is released, and the process may be linked to energy production (8). Thus, it is likely that the dissimilatory reduction of NO_3^- to NH_4^+ actually does occur to a significant extent in NH_4^+-rich sediments, but to the author's knowledge, this NH_4^+-producing pathway has never been quantified in marine sediments.

The capacity of a marine sediment for NO_3^- reduction by the two pathways was measured in the present study, in which sediment samples were incubated anaerobically with ^{15}N-labeled NO_3^- in closed bottles, and the production of labeled N_2 (N_2O) and NH_4^+ was followed.

The denitrification capacity was measured by the $^{15}N_2$ gas production in an assay where emission spectrophotometry was applied. Alternatively, denitrification was also estimated by the N_2O production in other sediment samples where the "acetylene blockage technique" was applied. This technique was based on the findings of Fedorova et al. (5), who noted that the ultimate reduction of N_2O to N_2 in the denitrification pathway could be blocked by C_2H_2, resulting in accumulation of N_2O. The "acetylene blockage technique" was applied in culture studies by Balderston et al. (1) and Yoshinari and Knowles (21) and in soils by Yoshinari et al. (20). Their results suggested that it is a useful method for the measurement of denitrification activity, since N_2O production could be easily assayed by gas chromatography (2, 9). The applicability of the acetylene blockage technique in marine sediments was tested in the present study, in which the technique was compared to a $^{15}N_2$ production assay.

The measured reduction capacities should provide information about the possible occurrence of the two reductive pathways in situ. The depth distribution of the reduction capacities in the sediment was recorded to get information about the types of bacteria involved.

MATERIALS AND METHODS

Sediment samples. Sediment samples were taken with a "Haps" corer (14) in April, 1977, at a location in the Limfjorden, Denmark, corresponding to station no. 5 of Jørgensen (12). The water depth was 10 m, and the bottom water temperature was 5 to 7°C. At this site, the salinity was 26‰. Subcores 15 cm in length were taken from the Haps core with 3.5-cm-wide Plexiglas tubes and stored in the laboratory at in situ temperature in the dark. Experiments were initiated within the following 3 days.

Incubations. Immediately before the incubations

were made, the redox potential profile was recorded in a sediment core. A platinum electrode was pushed stepwise into the sediment from above, and the redox potential was read after 1 min. Sediment density and porosity were determined by weighing known volumes of sediment, which were then dried to constant weight at 110°C.

A whole core was mixed for experiment A, which was a control experiment in which the assays for N_2 and N_2O production by denitrification were compared. The upper 12 cm of other cores was cut into four 3-cm segments for experiment B, in which $N_2(N_2O)$ and NH_4^+ production rates were followed. All segments were mixed thoroughly under N_2 in beakers before portions of 5 g were transferred into four series of 15-ml serum bottles. Each bottle was then purged extensively with O_2-free N_2 and finally stoppered with a greased (Ramsay vacuum grease) butyl rubber cap.

Pure C_2H_2 was injected in 100-μl quantities into each of the bottles which were assayed for N_2O production in the two experiments. The injected volume gave about 0.7% C_2H_2 in the gas phase of the bottles. Acetylene was omitted from the bottles for the $^{15}N_2$ production assay in experiment A.

Finally, 100 μl of an $Na^{15}NO_3$ (96.3% ^{15}N; VEB, Berlin) solution was injected, giving about 1.5 μmol of NO_3^-/cm^3 of sediment in the bottles, and thoroughly mixed into the sediment on a Vortex mixer before the bottles were incubated in the dark at 5°C.

The unlabeled products of reduction of native NO_3^- were neglected, since native NO_3^- in the upper segments was less than 10% of the added $^{15}NO_3^-$. Thus, the NO_3^- pool and the pools of the intermediates NO_2^-, NO, and N_2O in denitrification were considered to be fully ^{15}N labeled, and no attempt was made to discriminate between ^{14}N and ^{15}N in the assays for these compounds. Only for the production of N_2 and NH_4^+ was a discrimination necessary, since the native pools were high.

Gas analysis. A new bottle was used for each gas analysis to avoid multiple penetration of the rubber cap. The tightness of the caps was tested, and the loss of C_2H_2 from 15-ml, N_2-purged bottles without sediment was only 2.5%/day.

In experiment A, the rate of denitrification was eventually measured by the rate of $^{15}N_2$ production in that series of bottles where C_2H_2 was omitted. Using a Pressure-Lok gas-tight syringe, a 15-μl gas sample was taken from each bottle for ^{15}N analysis in a Statron NOI 4 ^{15}N analyzer. The needle of the locked syringe was inserted through a rubber septum (Hamilton, red) into a short side arm emerging from a three-way stopcock which also joined a vacuum line and the discharge tube in the analyzer. Evacuation of the system for 5 min removed the previous gas sample and excluded air contamination. The vacuum was better than 13.3 Pa when the vacuum line was closed off. Finally the syringe was unlocked, and the gas sample was injected and analyzed. Water vapor was removed by 0.5 g of "Drierite" (8 mesh) which was placed between two cotton plugs in the injection arm. The injected gas volume of 15 μl was found to give optimal intensity of the discharge. The minimal detectable ^{15}N enrichment in the gas samples was 0.2%. The measured ^{15}N abundance in pure N_2 from a cylinder was used as a standard of reference and subtracted from the values measured in the incubated bottles.

In the same experiment, this assay for $^{15}N_2$ production was compared to the acetylene blockage technique which was used in a second series of bottles with $^{15}NO_3^-$-amended sediment and 0.7% C_2H_2. The N_2O production rate was measured by gas chromatography. At intervals, 50-μl gas samples were taken from the bottles and assayed on a Packard Becker 417 gas chromatograph with a thermal conductivity detector operated at 140°C and a bridge current of 300 mA. On the measuring side was a 2-m by 0.32-m cm column of Porapak Q. He carrier flow was 20 ml/min. Full-scale deflection at 1 mV on the HP 7100 BM recorder corresponded to 20 nmol of N_2O, and minimal detectable N_2O concentration in the gas phase of the bottles was 0.06%.

The acetylene blockage technique described was also applied in experiment B.

The concentration of $^{15}N_2$ and N_2O in the bottles was expressed as μmol of N/cm^3 of wet sediment, and the estimated production rates were expressed as μmol of N/cm^3 per day. Correction was made for the solubility of N_2O in water.

Chemical assays. In experiment B, colorimetric assays for NO_3^-, NO_2^-, and total NH_4^+ were made at intervals. The sediment samples were killed with chloroform (200 μl/5 g of sediment). A 2-h extraction at 5°C with 0.5 M KCl (5 ml/5 g of sediment) was performed before the bottles were centrifuged at 2,000 × *g* for 10 min. A subsample of supernatant from each bottle was immediately removed and frozen until the chemical analyses were made.

Total NH_4^+ (soluble, interstitial NH_4^+ + adsorbed, exchangeable NH_4^+) was determined by the phenol-hypochlorite method of Solórzano (17). The ^{15}N content of this pool was measured on the ^{15}N analyzer, using the technique of Fiedler and Proksch (6) for preparation of the discharge tubes and the microdiffusion technique of Conway (4) for preparation of the NH_4^+-containing capillaries. Assays for NO_2^- were made by the method of Strickland and Parsons (18), and assays for NO_3^- were made by the modified brucine method of Kahn and Brezenski (13).

All concentrations were expressed as μmol of N/cm^3 of wet sediment, and $^{15}NH_4^+$ production rates were expressed as μmol of N/cm^3 per day.

RESULTS

Sediment characteristics. Some basic characteristics of the sediment are given in Table 1. The pronounced gradients for the redox potential and the NO_3^- concentration indicate that in situ O_2 and NO_3^- respiration were localized in the upper few centimeters of the sediment.

No method was available for the measurement of O_2 concentrations in the sediment, but it was anticipated from the extension of the brown-colored surface zone (precipitates of hydrous iron oxides) that O_2 penetrated about 2 cm into the sediment in situ.

The total NH_4^+ concentrations were high compared with the NO_3^- and NO_2^- concentra-

TABLE 1. *Sediment characteristics*

Sediment segment (cm)	Porosity (% [wt/wt])	Density (g/cm³)	Eh (mV)	NO_3^- (μmol of N/cm³)	NO_2^- (μmol of N/cm³)	Total NH_4^+[a] (μmol of N/cm³)
0–3	76	1.10	+75	0.09	0.05	1.15
3–6	72	1.18	−225	0.01	trace	1.75
6–9	69	1.21	−250	—[b]	—[b]	1.85
9–12	65	1.20	−275	—[b]	—[b]	1.90

[a] Soluble NH_4^+ + exchangeable NH_4^+.
[b] Not detectable.

tions. Exchangeable NH_4^+ accounted for 50% of the total NH_4^+; this was a constant factor in the present sediment (T. H. Blackburn, unpublished results).

Gas production assays. The N_2 production rates in experiment A were based solely on the increments of the ^{15}N content in the N_2 gas phase of the incubated bottles, since the native, unlabeled NO_3^- pool was neglected. Any NO and N_2O that might have been produced was disregarded, since these gases only appeared transiently in trace amounts during an early stage of incubation. Isotopic fractionation in the denitrification process was also neglected in this context, because the reported fractionation factors are less than 1.023 (3).

Figure 1 shows the production of $^{15}N_2$ in the absence of C_2H_2 and the production of N_2O in the presence of 0.7% C_2H_2 in the two series of bottles in experiment A. The rates of $^{15}N_2$ and N_2O production were similar. Gas production ceased at the time when NO_3^- was ultimately exhausted, and equal gas accumulations were obtained in the two series. It was concluded that significant $^{15}N_2$ production was absent in the bottles with C_2H_2 and that the reduction of N_2O to N_2 was completely blocked in the presence of 0.7% C_2H_2. The data showed that acetylene blockage technique was applicable to the present measurements of denitrification capacities in marine sediment.

The acetylene blockage technique was preferred in experiment B, since the gas chromatographic assay for N_2O production was more rapid and sensitive than the $^{15}N_2$ production assay.

Denitrification. The disappearence of NO_3^- and the production of N_2O in experiment B is shown for the four sediment segments in Fig. 2. The rate of NO_3^- disappearence decreased with depth in the sediment. Transient accumulation of NO_2^- was observed in the two upper segments, whereas only trace amounts of NO could be detected in the bottles by the gas chromatographic assay. The production of N_2O began within 3 h after addition of NO_3^- in the two surface segments, whereas increasing delays were observed in the two deeper segments. The

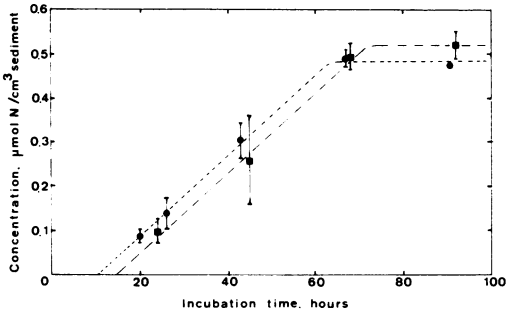

FIG. 1. *Production of N_2O (●) from NO_3^- respiration in the presence of 0.7% C_2H_2, and production of N_2 (■) from NO_3^- respiration in the absence of C_2H_2.*

N_2O production rates were apparently constant in all segments after the initial time lags when N_2O production was not detectable by the gas chromatographic assay, but sufficient points were not available to preclude an exponential increase. The presence of long time lags before N_2O production could be measured implied a risk of significant cell growth in the incubated bottles, and so the measured denitrification capacities in the two deeper segments were possibly overestimated.

The denitrification capacities in the segments were estimated as the maximal rates of N_2O production and are given in Table 2. The values ranged from 0.87 to 0.10 μmol of N/cm^{-3} per day in the sediment.

Reduction of NO_3^- to NH_4^+. The production of $^{15}NH_4^+$ in the four segments is also shown in Fig. 2. In all segments, the production of $^{15}NH_4^+$ began immediately after addition of NO_3^-. Since the production rates declined in the upper segments after the initial 5 to 10 h, the estimated capacities were based on the steady initial rates. The values are given in Table 2.

Decreasing values were found with depth in the sediment as in the case of denitrification, and the measured range was of similar magnitude, from 0.75 μmol of N/cm^3 per day in the top segment to 0.12 μmol of N/cm^3 per day in the deepest segment.

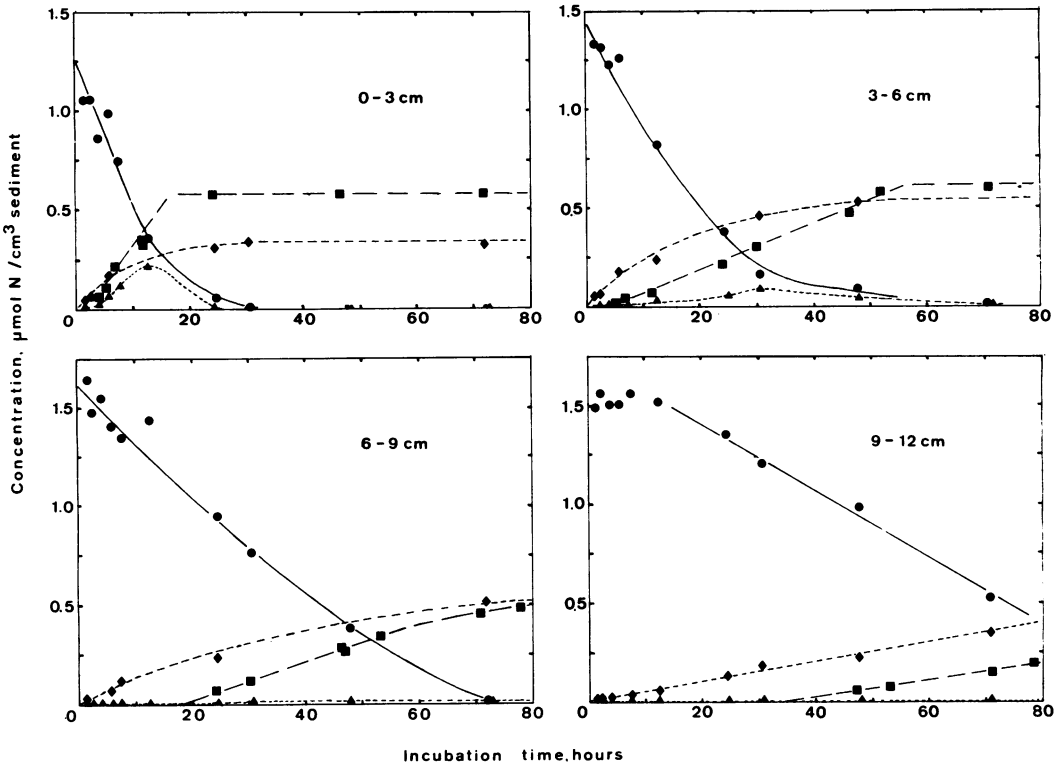

FIG. 2. *Concentration of NO_3^- (●), NO_2^- (▲), N_2O (■), and NH_4^+ (◆) during anaerobic incubation of NO_3^--amended sediment segments with 0.7% C_2H_2. Segment depths are indicated.*

TABLE 2. *Measured capacities for denitrification and nitrate reduction to ammonia*

Sediment segment (cm)	$NO_3^- \rightarrow N_2$ (N_2O) (µmol of N/cm³ per day)	$NO_3^- \rightarrow NH_4^+$ (µmol of N/cm³ per day)
0–3	0.87	0.75
3–6	0.27	0.57
6–9	0.23[a]	0.43
9–12	0.10[a]	0.12

[a] Possibly overestimated due to cell growth during incubation.

DISCUSSION

Distribution of denitrifying activity. In the investigated sediment, in situ O_2 and NO_3^- respiration could take place only within the upper 2 and the upper 6 cm, respectively, as revealed by the absence of O_2 and NO_3^- below these depths. Concurrent O_2 and NO_3^- respiration might occur, however, in the upper 2 cm if reduced microniches were present in this "oxidized" zone. Jørgensen (11) demonstrated SO_4^{2-} reduction, a strictly anaerobic process, in an oxidized zone. It was anticipated from this observation that denitrification was also likely to occur in this zone.

Throughout the year, the vertical distribution of NO_3^- changes by several centimeters in the sediment, but its presence is always restricted to the upper 5 to 6 cm (K. Henriksen, unpublished results). It is likely, however, that migrations of the bacteria and bioperturbation by burrowing animals may take denitrifying bacteria down to the deeper, NO_3^--deficient layers. The bacteria would only be able to grow there, however, if they were also facultative fermenters. Possibly, bacteria which are capable of anaerobic fermentation and are also potential denitrifiers, e.g., some bacilli (7), are present in the deeper layers of these sediments.

Denitrification capacity. The potential for denitrification was, due to the exclusion of the alternative O_2 respiration, an indication of the abundance of the denitrifying bacteria. The high capacities which were measured in the upper two sediment segments confirmed that in situ denitrification was restricted to a few centimeters of surface sediment. The capacities which were measured in the two deeper segments were significant but possibly overestimated due to proliferating populations during the prolonged incubations. The denitrifying activity which did

occur in these layers was probably due to bacteria of a type which was fermenting in situ but was able to respire when supplied with NO_3^-.

Occurrence of other NO_3^- reducers. The assimilatory reduction, in which NH_4^+ is incorporated into cell material, is usually repressed by high concentrations of NH_4^+. A few exceptions do exist, however; e.g., some clostridia where the enzyme synthesis is constitutive (15). If fermenters like the clostridia do reduce NO_3^- to NH_4^+ in an NH_4^+-rich environment like the present sediment, the process may have an energetic rather than a nutritional value. The studies of Ishimoto and Egami (10) and Takahashi et al. (19) indicated that energy production was indeed linked to the reduction of NO_3^- to NH_4^+ by some clostridia, and Hasan and Hall (8) showed that the presence of NO_3^- enhanced ATP production in *Clostridium perfringens*. The process was clearly dissimilative, since NH_4^+ was released, and was also of fermentative character, since substrate-level phosphorylation was involved.

Constitutive enzyme synthesis in the NH_4^+-producing pathway was also demonstrated (16) for a denitrifying aerobe, *Paracoccus denitrificans*, as quoted by Payne (15).

Such capable fermenters and respirers were likely to cause the observed $^{15}NH_4^+$ production in the sediment under study.

Sediment capacity for reduction of NO_3^- to NH_4^+. The measured capacities for this reductive pathway were of the same magnitude as those recorded for denitrification, and since the $^{15}NH_4^+$ production was also spontaneous in the deeper segments, it was evident that significant populations of capable bacteria were present in all layers.

The experiments suggested that the reduction of NO_3^- to NH_4^+ may be quantitatively important in marine sediments.

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LITERATURE CITED

1. **Balderston, W. L., B. Sherr, and W. J. Payne.** 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. **31**:504–508.
2. **Barbaree, J. M., and W. J. Payne.** 1967. Products of denitrification by a marine bacterium as revealed by gas chromatography. Mar. Biol. **1**:136–139.
3. **Blackmer, A. M., and J. M. Bremner.** 1977. Nitrogen isotope discrimination in denitrification of nitrate in soils. Soil Biol. Biochem. **9**:73–77.
4. **Conway, E. J.** 1947. Microdiffusion analysis and volumetric error. Crosby Lockwood and Son Ltd., London.
5. **Fedorova, R. I., E. I. Milekhina, and N. I. Il'Yukhina.** 1973. Evaluation of the method of "gas metabolism" for detecting extraterrestrial life. Identification of nitrogen-fixing microorganisms. Akad. Nauk SSSR Izvestia Ser. Biol. **6**:797–806.
6. **Fiedler, R., and G. Proksch.** 1975. The determination of nitrogen-15 by emission and mass spectrometry in biochemical analysis: a review. Anal. Chim. Acta **78**:1–62.
7. **Gibson, T., and R. E. Gordon.** 1974. Genus *Bacillus Cohn 1872, 174*, p. 529–550. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Hasan, S. M., and J. B. Hall.** 1975. The physiological function of nitrate reduction in *Clostridium perfringens*. J. Gen. Microbiol. **87**:120–128.
9. **Hollis, O. L.** 1966. Separation of gaseous mixtures using porous polyaromatic beads. Anal. Chem. **38**:309–316.
10. **Ishimoto, M., and F. Egami.** 1959. Meaning of nitrate and sulfate reduction in the process of metabolic evolution, p. 555–561. *In* F. Clark and R. L. M. Synge (ed.), Proceedings of the first international symposium on the origin of life on earth (1957). Pergamon Press, New York.
11. **Jørgensen, B. B.** 1977. Bacterial sulfate reduction within reduced microniches of oxidized marine sediments. Mar. Biol. **41**:7–17.
12. **Jørgensen, B. B.** 1977. Distribution of colorless sulfur bacteria (*Beggiatoa* spp.) in a coastal marine sediment. Mar. Biol. **41**:19–28.
13. **Kahn, L., and F. T. Brezenski.** 1967. Determination of nitrate in estuarine waters. Environ. Sci. Technol. **1**:488–491.
14. **Kanneworff, E., and W. Nicolaisen.** 1973. The "Haps," a frame supported bottom corer. Ophelia **10**:119–128.
15. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409–452.
16. **Pichinoty, F.** 1970. Les nitrates-reductases bacteriennes. IV. Regulation de la biosynthese et de l'activite de l'enzyme B. Arch. Microbiol. **71**:116–122.
17. **Solórzano, L.** 1969. Determination of ammonia in natural waters by the phenylhypochlorite method. Limnol. Oceanogr. **14**:799–801.
18. **Strickland, J. D. H., and T. R. Parsons.** 1968. A practical handbook of sea water analysis. Fish. Res. Board Can., Bull. 167.
19. **Takahashi, H., S. Tanaguchi, and F. Egami.** 1963. Inorganic nitrogen compounds distribution and metabolism, p. 91–202. *In* M. Florkin and H. S. Mason (ed.), Comparative biochemistry. Academic Press Inc., New York.
20. **Yoshinari, T., R. Hynes, and R. Knowles.** 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. **9**:117–183.
21. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705–710.